

## Separation of NH and NH<sub>2</sub> Resonances in <sup>1</sup>H-Detected Heteronuclear Multiple-Quantum Correlation Spectra

LEWIS E. KAY AND AD BAX

*Laboratory of Chemical Physics, NIDDK, National Institutes of Health, Bethesda, Maryland 20892*

Received December 12, 1988

<sup>15</sup>N-<sup>1</sup>H heteronuclear multiple-quantum correlation spectroscopy (HMQC) (1, 2) is a useful technique for facilitating the sequential assignment of proteins with <sup>1</sup>H NMR spectra that are too complex for analysis by conventional homonuclear experiments alone (3, 4). The <sup>15</sup>N-<sup>1</sup>H HMQC experiment is particularly useful for the study of proteins in the molecular weight range 10-20 kDa since the transfer of magnetization between an amide proton and its coupled <sup>15</sup>N partner proceeds via a scalar coupling constant that is much larger than typical <sup>1</sup>H linewidths (5). Moreover, as has been discussed previously, heteronuclear multiple-quantum relaxation time constants are independent of the large <sup>15</sup>N-<sup>1</sup>H dipolar interaction (6), to a first-order approximation, and hence tend to be longer than the corresponding amide proton transverse relaxation times. This may be particularly important for applications to proteins with molecular weights in excess of 15-20 kDa where short transverse relaxation times, and not multiple-bond homonuclear and heteronuclear scalar couplings or digitization, will determine spectral resolution. The HMQC experiment is most efficiently carried out on molecules uniformly labeled with <sup>15</sup>N, a relatively simple and inexpensive process for cloned proteins that are expressed in a microorganism (5, 7).

Despite the substantial chemical-shift dispersion in both the <sup>15</sup>N and the <sup>1</sup>H dimensions of the <sup>15</sup>N-<sup>1</sup>H HMQC spectrum, some resonance overlap is present nevertheless, especially for application to proteins with molecular weights in excess of 10 kDa. In addition, the presence of both NH and NH<sub>2</sub> resonances can complicate spectral interpretation. In this Note, a simple scheme for the separation of NH and NH<sub>2</sub> resonances in HMQC spectra is described, which simplifies the spectra.

Figure 1a illustrates the conventional HMQC pulse sequence for recording <sup>15</sup>N-<sup>1</sup>H correlated spectra in water. Figures 1b and 1c indicate the pulse schemes that are employed to separate NH and NH<sub>2</sub> resonances. In all cases the carrier is positioned in the center of the NH region, necessitating the use of an off-resonance water presaturation scheme (8, 9). Separation of NH and NH<sub>2</sub> moieties is achieved by exploiting the fact that isolated NH resonances are not modulated by the <sup>15</sup>N-<sup>1</sup>H scalar coupling during multiple-quantum evolution. In contrast, for NH<sub>2</sub> groups the scalar coupling between the <sup>15</sup>N spin and the <sup>1</sup>H spin that does not participate in the multiple-quantum transition (passive spin) results in a modulation of the multiple-quantum signal (10). This modulation of the evolution of the NH multiple-quantum coherence pro-

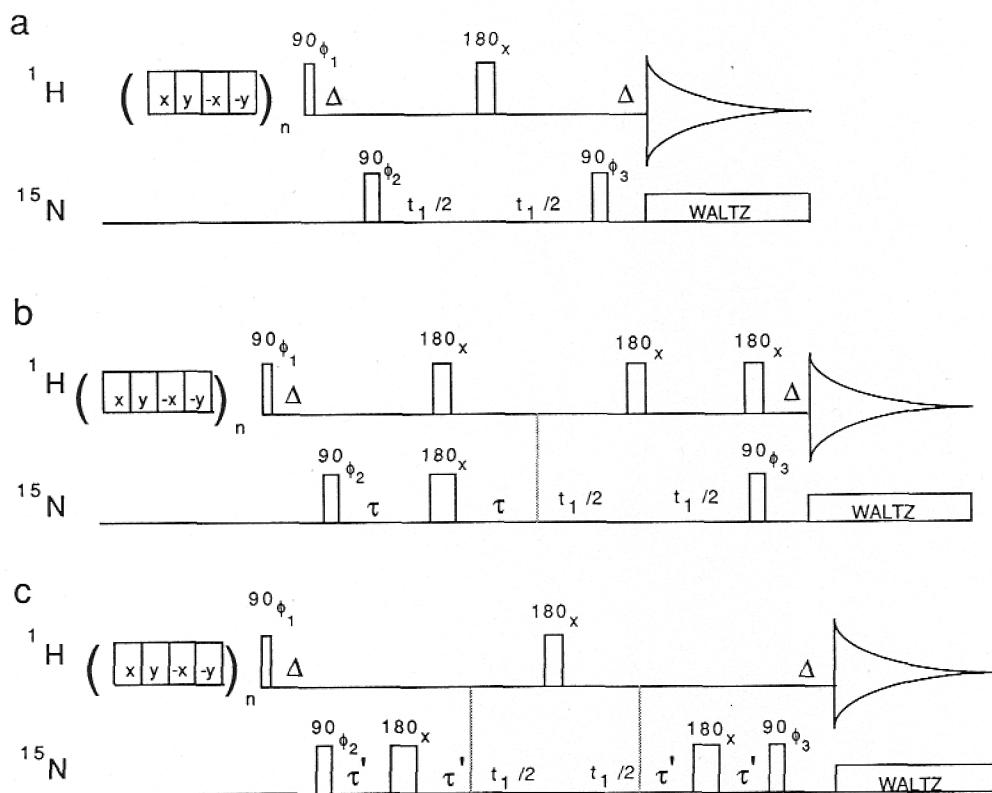


FIG. 1. (a) The conventional pulse scheme used to record  $^{15}\text{N}$ - $^1\text{H}$  HMQC experiments; (b, c) the scheme used to separate NH from  $\text{NH}_2$  resonances in an  $^{15}\text{N}$ - $^1\text{H}$  correlation map. The phase cycling employed is as follows:  $\phi_1 = 4(x), 4(y), 4(-x), 4(-y)$ ;  $\phi_2 = x, y, -x, -y$ ;  $\phi_3 = 16(x), 16(-x)$ ;  $\text{ACQ} = 2(x), 2(-x), 2(-y), 2(y), 2(-x), 2(x), 2(y), 2(-y)$ , with data for odd and even numbered scans stored separately. The phase of the receiver is inverted every 16 scans. To minimize relaxation losses, the value of  $\Delta$  is set to 4.5 ms, slightly less than  $1/(2J_{\text{NH}})$ . The value of  $\tau$  is set to  $1/(2J_{\text{NH}})$  so that  $^1\text{H}$  magnetization from  $\text{NH}_2$  resonances is inverted relative to  $^1\text{H}$  magnetization from NH resonances. The value of  $\tau'$  is set to  $1/(4J_{\text{NH}})$  so that sequences b and c have the same duration. To minimize data storage space, the carrier is positioned in the center of the amide resonances and an off-resonance DANTE-type water suppression scheme (8, 9) is employed. Depending on the spectrometer convention, the phases of the off-resonance presaturation pulses may have to be  $(x, y, -x, -y)$  or  $(x, -y, -x, y)$ .

ceeds in a manner completely analogous to the modulation of transverse magnetization in a homonuclear two-spin system, caused by the scalar coupling between the two spins. Therefore, by setting  $\tau = 1/(2J_{\text{N-H}}) \approx 5.8$  ms in the sequence of Fig. 1b, cross peaks associated with  $\text{NH}_2$  resonances will have opposite phase relative to cross peaks correlating  $^{15}\text{N}$  and  $^1\text{H}$  shifts from NH resonances. The sequence indicated in Fig. 1c refocuses the effects of the scalar modulation of the multiple-quantum coherence so that cross peaks associated with both NH and  $\text{NH}_2$  resonances have the same phase. The value of  $\tau'$  is set to  $1/(4J_{\text{N-H}})$  in this case so that relaxation effects during the multiple-quantum periods in both sequences are identical. Subtraction of the spectra generated from the sequences of Figs. 1b and 1c generates an  $^{15}\text{N}$ - $^1\text{H}$  correla-

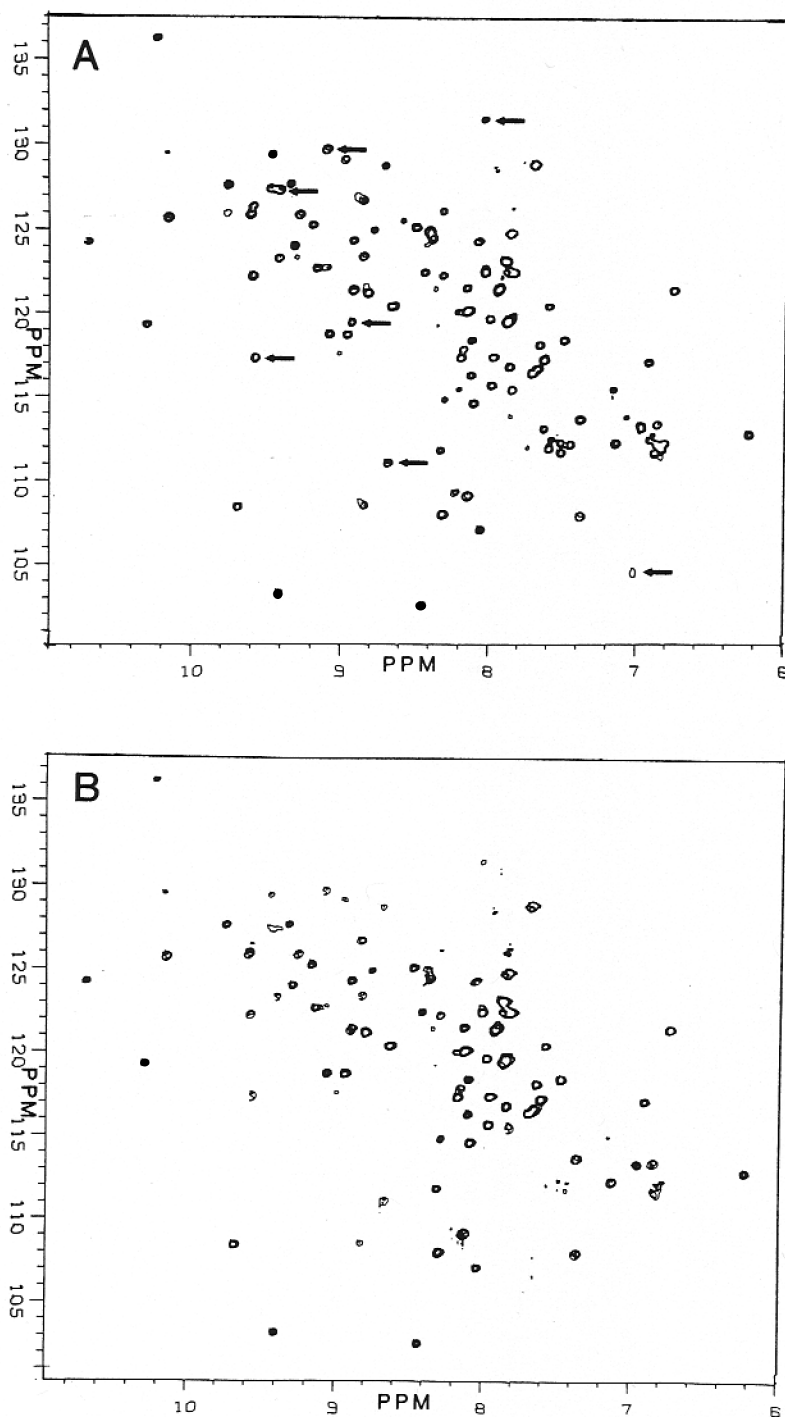


FIG. 2. (A) The 500 MHz  $^{15}\text{N}$ - $^1\text{H}$  HMQC correlation spectrum of *S. Nase* recorded at 37°C with the sequence of Fig. 1a. In (B) and (C) are shown  $^{15}\text{N}$ - $^1\text{H}$  maps of NH correlations (B) and  $\text{NH}_2$  correlations (C) obtained by adding and subtracting the spectra recorded with the sequences of Figs. 1b and 1c, respectively; 160  $t_1$  increments were obtained for each spectrum with 256 scans per  $t_1$  point. The spectra were recorded on a modified NT 500 MHz spectrometer. Several of the NH correlations in (A) that decrease in intensity or have disappeared in (B) are indicated with arrows.

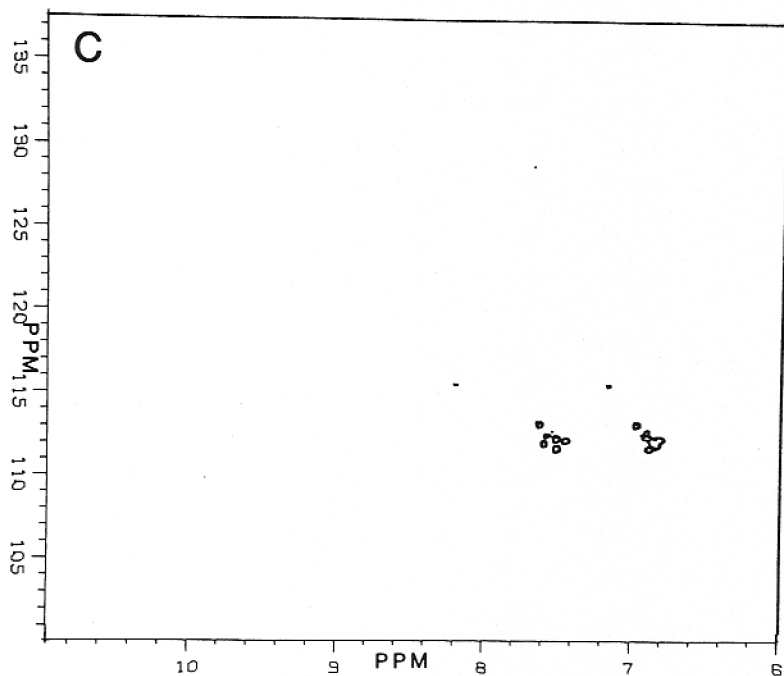
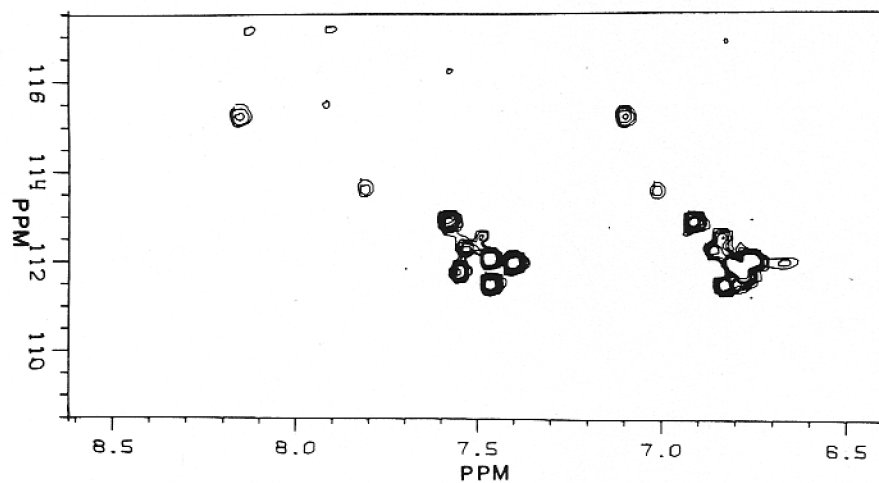


FIG. 2—Continued

tion map displaying  $\text{NH}_2$  resonances only, while addition of the spectra produces a map of the  $\text{NH}$  resonances.

Figure 2A presents a regular HMQC  $^{15}\text{N}$ - $^1\text{H}$  correlation map of the protein staphylococcal nuclease (S. Nase) at a concentration of 1.8 mM, complexed with pdTp and

FIG. 3. Expanded region of the  $^{15}\text{N}$ - $^1\text{H}$  map of Fig. 2C showing the  $\text{NH}_2$  correlations.

$\text{Ca}^{2+}$  and dissolved in 90%  $\text{H}_2\text{O}$ /10%  $\text{D}_2\text{O}$  at pH 6.5, 37°C. The protein is uniformly labeled with  $^{15}\text{N}$ . Figures 2B and 2C present correlation maps of the NH and  $\text{NH}_2$  resonances in S. Nase, respectively. Comparison of Figs. 2A and 2B indicates that in some cases cross peaks are greatly attenuated in the edited NH spectrum. This is a result of the transverse relaxation that proceeds during the  $\tau$  and  $\tau'$  periods in the sequences of Figs. 1b and 1c. Several of these correlations are indicated with arrows in Fig. 2A. These resonances are presumably broadened because of an exchange of different conformations of the protein at a rate that is intermediate on the NMR time scale (11). As mentioned above, the durations of both sequences 1b and 1c are identical, and hence the absolute intensities of spectra recorded with the schemes of Figs. 1b and 1c are the same. The difference of these spectra gives, therefore, an  $\text{NH}_2$  correlation map with a minimal number of subtraction artifacts.

Figure 3 presents an expanded plot of the  $^{15}\text{N}$ - $^1\text{H}$  correlation map illustrated in Fig. 2C. Correlations are observed for approximately half of the  $\text{NH}_2$  sites in the protein. The intensities of these correlations vary significantly, suggesting different degrees of mobility, transverse relaxation rates influenced by solvent exchange, or two-site chemical exchange caused by 180° degree flips about the C-N bond.

Several schemes for the separation of resonances from  $\text{AX}$ ,  $\text{AX}_2$ , and  $\text{AX}_3$  spin systems in two-dimensional heteronuclear correlation spectra have been proposed based on INEPT (12) or DEPT (13) sequences. The earliest versions of these experiments involved direct detection of the low  $\gamma$  nucleus (14-16). While the sensitivity of these experiments is improved by the fact that proton polarization is transferred to the heteronucleus, the experiments are still on the order of  $(\gamma_{\text{A}}/\gamma_{\text{X}})^{1.5}$  less sensitive than the HMQC experiment, where  $\gamma_{\text{A}}$  and  $\gamma_{\text{X}}$  are the magnetogyric ratios of the heteronucleus and the proton, respectively. An alternative editing strategy, based on a double transfer of polarization (17), has recently been employed by Wagner and co-workers (18). These workers recorded edited heteronuclear experiments using a double DEPT editing sequence to separate  $\text{CH}$ ,  $\text{CH}_2$ , and  $\text{CH}_3$  resonances. While these experiments enjoy increased sensitivity over the earlier experiments, the large number of pulses and the overall length of the pulse sequence can be prohibitive, especially for large molecules with short transverse relaxation times. Moreover, for  $\text{CH}_2$  and  $\text{NH}_2$  spin systems, only one-half of the total proton magnetization returns for detection (19). In contrast, the HMQC scheme that we have proposed uses the full proton magnetization of  $\text{CH}_2$  and  $\text{NH}_2$  groups. In addition, the HMQC scheme minimizes the number of pulses and the overall length of the sequences.

In summary, we have presented a simple method for the separation of NH and  $\text{NH}_2$  resonances in a heteronuclear correlation map. This results in a simplification of the HMQC spectrum and serves as an aid in the assignment of cross peaks. The method can, of course, be applied to  $^{13}\text{C}$ - $^1\text{H}$  HMQC experiments as well.

#### ACKNOWLEDGMENTS

We thank Rolf Tschudin for technical support, Dennis A. Torchia and Steven W. Sparks for providing us with the sample of S. Nase, and Julie Forman-Kay for a critical reading of the manuscript. This work was supported by the Intramural AIDS Antiviral Program of the Office of the Director of the National Institutes of Health. L.E.K. acknowledges financial support from the Medical Research Council of Canada and the Alberta Heritage Trust Foundation.



## REFERENCES

1. M. R. BENDALL, D. T. PEGG, AND D. M. DODDREL, *J. Magn. Reson.* **52**, 81 (1983).
2. A. BAX, R. H. GRIFFEY, AND B. L. HAWKINS, *J. Magn. Reson.* **55**, 301 (1983).
3. D. A. TORCHIA, S. W. SPARKS, AND A. BAX, *Biochemistry* **27**, 14 (1988).
4. A. M. GRONENBORN, A. BAX, P. T. WINGFIELD, AND G. M. CLORE, *FEBS Lett.* **1**, 93 (1989).
5. L. P. MCINTOSH, R. H. GRIFFEY, D. C. MUCHMORE, C. P. NIELSON, A. G. REDFIELD, AND F. W. DAHLQUIST, *Proc. Natl. Acad. Sci. USA* **84**, 1244 (1987).
6. A. BAX, L. E. KAY, S. W. SPARKS, AND D. A. TORCHIA, *J. Am. Chem. Soc.* **111**, 408 (1989).
7. M. A. WEISS, A. JEITLER-NILSSON, N. J. FISCHBEIN, M. KARPLUS, AND R. T. SAUER, In "NMR in the Life Sciences" (E. M. Bradbury and C. Nicolini, eds.), pp. 37-48, NATO ASI Series, Vol. 107, Plenum, London, 1986.
8. D. MARION, L. E. KAY, S. W. SPARKS, D. A. TORCHIA, AND A. BAX, *J. Am. Chem. Soc.* **111**, 1515 (1989).
9. L. E. KAY, D. MARION, AND A. BAX, *J. Magn. Reson.* **84**, 72 (1989).
10. O. W. SØRENSEN, G. W. EICH, M. H. LEVITT, G. BODENHAUSEN, AND R. R. ERNST, *Prog. NMR Spectrosc.* **16**, 163 (1983).
11. J. I. KAPLAN AND G. FRAENKEL, "NMR of Chemically Exchanging Systems," Academic Press, New York, 1980.
12. G. A. MORRIS AND R. FREEMAN, *J. Am. Chem. Soc.* **101**, 760 (1979).
13. D. M. DODDRELL, D. T. PEGG, AND M. R. BENDALL, *J. Magn. Reson.* **48**, 323 (1982).
14. M. H. LEVITT, O. W. SØRENSEN, AND R. R. ERNST, *Chem. Phys. Lett.* **94**, 540 (1983).
15. M. R. BENDALL AND D. T. PEGG, *J. Magn. Reson.* **53**, 144 (1983).
16. T. T. NAKASHIMA, B. K. JOHN, AND R. E. D. MCCLUNG, *J. Magn. Reson.* **59**, 124 (1984).
17. G. BODENHAUSEN AND D. J. RUBEN, *Chem. Phys. Lett.* **69**, 1 (1980).
18. V. THANABAL AND G. WAGNER, "29th Experimental NMR Conference, Rochester, New York, April, 1988," Abstract No. 71.
19. M. F. SUMMERS, L. G. MARZILLI, AND A. BAX, *J. Am. Chem. Soc.* **108**, 4285 (1986).